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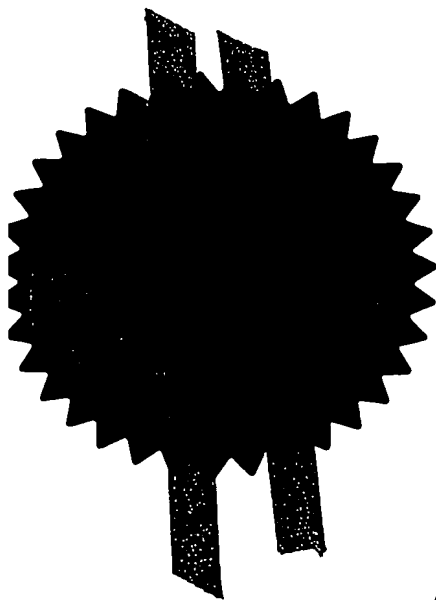
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UNITED KINGDOM

Patents ADP number *(if you know it)*

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ENGLAND AND WALES

4. Title of the invention

A PROTEIN INVOLVED IN CARCINOMA

5. Name of your agent *(if you have one)*

BLAKEY, ALISON JANE
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

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Description 21

Claim(s) 3

Abstract

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A PROTEIN INVOLVED IN CARCINOMA

This invention provides a new use for protein-tyrosine kinase-related receptor (PTK7), in the prophylaxis, diagnosis, and/or treatment of carcinoma. The invention further provides pharmaceutical compositions comprising PTK7, including vaccines, antibodies that recognise PTK7 and agents that modulate the expression and/or activity of PTK7 or that modulate the expression of nucleic acids encoding PTK7. These compositions, antibodies and agents are useful in the diagnosis, prophylaxis and/or treatment of carcinoma.

Tumour specific proteins have been identified for a number of cancer types using techniques such as differential screening of cDNAs (Hubert, R.S., et al., 1999, *Proc. Natl. Acad. Sci. USA* 96, 14523-14528) and the purification of cell-surface proteins that are recognised by tumour-specific antibodies (Catimel, B., et al., 1996, *J. Biol. Chem.* 271, 25664-25670). More recently, DNA 'chips' containing up to 10,000 expressed sequence elements have been used to characterise tumour cell gene expression (Dhanasekaran, S.M., et al., 2001, *Nature* 412, 822-826). However, there are several reasons why the numerous and extensive previous transcriptomic analysis of breast cancer may not have revealed all, or even most, tumour associated proteins. These include; (i) a lack of correlation between transcript and disease-associated protein levels, particularly common for membrane proteins that often have a long half-life and as such do not have a high mRNA turnover. Therefore, whilst the difference in protein levels between normal and cancerous cells are consistent it is often difficult to associate changes in the mRNA for a given membrane protein with the cancerous state. (ii) Translocation of a protein in the disease state rather than simply differential levels of the transcript, for example, erbB2/HER2-neu, shows much greater plasma-membrane localisation in cancer cells than normal breast cells, and the transcription factors oestrogen receptor and STAT3 translocate to the nucleus to exert their tumourigenic effects. (iii) Novel/uncharacterised genes are not highly represented within the 'closed system' of a cDNA array where there are restrictions on the number of expressed sequence elements per chip and the knowledge and availability of DNA clones.

The term 'carcinoma' refers to a malignant new growth that arises from epithelium, found in skin or, more commonly, the lining of body organs, for example: breast, prostate, lung, kidney, pancreas, stomach or bowel. Carcinomas tend to infiltrate into adjacent tissue and spread (metastasise) to distant organs, for example: to bone, liver, lung or the brain.

The present invention relates to a novel tumour antigen.

Briefly, PTK7 was isolated from lung and liver cancer cell membranes, purified by one-dimensional (1D) gel electrophoresis and characterised by mass spectrometry. Analysis of PTK7 mRNA expression revealed that it is upregulated in lung, kidney, breast and pancreatic cancer samples when compared to normal tissue, or matched control tissue. In addition, PTK7 showed a marked increase in expression in some cases of breast cancer with lymph node metastases compared to both samples from breast cancer patients with no lymph node metastases and control samples.

Accordingly, the present invention provides a method of screening for and/or diagnosis of carcinoma in a subject, and/or monitoring the effectiveness of carcinoma therapy, which

comprises the step of detecting and/or quantifying in a biological sample obtained from said subject:

- (i) a PTK7 polypeptide which:
 - a) comprises or consists of the amino acid sequence shown in Figure 1 (SEQ ID NO:1);
 - b) is a derivative having one or more amino acid substitutions, modifications, deletions or insertions relative to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1) which retains the activity of PTK7; or
 - c) is a fragment of a polypeptide having the amino acid sequence shown in Figure 1 (SEQ ID NO: 1), which is at least ten amino acids long and has at least 70% homology over the length of the fragment;

The above polypeptides are referred to as "PTK7 polypeptides". The term "polypeptides" includes peptides, polypeptides and proteins. These are used interchangeably unless otherwise specified; these terms include any of the PTK7 polypeptides as described in a)-c) above. Additionally, the term "PTK7 polypeptides", includes derivatives having one or more amino acid substitutions, modifications, deletions or insertions relative to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1) wherein the polypeptide is a dominant negative mutant of PTK7.

In a further embodiment, the level of the PTK7 polypeptide is compared to a reference range or control.

Preferably a derivative according to b) has at least 70% identity to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1), more preferably it has at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity. Percentage identity is a well known concept in the art and can be calculated using, for example but without limitation, the BLAST™ software available from NCBI (Altschul, S.F. *et al.*, 1990, J. Mol. Biol. 215:403-410; Gish, W. & States, D.J. 1993, Nature Genet. 3:266-272. Madden, T.L. *et al.*, 1996, Meth. Enzymol. 266:131-141; Altschul, S.F. *et al.*, 1997, Nucleic Acids Res. 25:3389-3402); Zhang, J. & Madden, T.L. 1997, Genome Res. 7:649-656).

Fragments of PTK7 polypeptides as described in c) above are at least 10 amino acids in length, preferably they are at least 20, at least 30, at least 50 or at least 100 amino acids in length. A fragment has at least 70% identity over its length to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1), more preferably it has at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity.

PTK7 polypeptides for use in the present invention can be prepared in any suitable manner. In one embodiment, PTK7 polypeptides are provided in isolated form and include PTK7 polypeptides that have been purified to at least some extent. PTK7 polypeptides can also be produced using recombinant methods, synthetically produced or produced by a combination of these methods. PTK7 polypeptides may be provided in substantially pure form, that is to say free, to a substantial extent, from other proteins. Thus, a PTK7 polypeptide may be provided in a composition in which it is the predominant component present (i.e. it is present at a level of at least 50%; preferably at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%; when determined on a weight/weight basis excluding solvents or carriers). PTK7

polypeptides may be in the form of the naturally occurring protein or may be part of a larger protein such as a precursor or a fusion protein. It can be advantageous to prepare a polypeptide comprising an additional amino acid sequence which contains a signal sequence, for example and without limitation, a signal peptide for secretion, or a leader sequence, a pro-sequence, a sequence to aid purification such as an affinity tag or an additional sequence conferring stability during production of the polypeptide. Such sequences may be optionally removed as required. Thus, a PTK7 polypeptide may be fused to other moieties including other polypeptides. Means for preparing such polypeptides are well known in the art. Recombinant polypeptides for use in the present invention can be prepared, for example, from genetically engineered host cells comprising expression vectors that are available commercially and are well known in the art; the expression vector comprises coding sequence for a PTK7 polypeptide. Alternatively, cell-free translation systems can be employed to produce recombinant polypeptides (e.g. rabbit reticulocyte lysate, wheat germ lysate, SP6/T7 *in vitro* T&T and RTS 100 *E. Coli* HY transcription and translation kits from Roche Diagnostics Ltd., Lewes, UK and the TNT Quick coupled Transcription/Translation System from Promega UK, Southampton, UK).

The methods of diagnosis according to the present invention may be performed using a number of methods known to those skilled in the art, including, without limitation, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, 2 dimensional gel electrophoresis, immunocytochemistry, immunohistochemistry, immunoassays, e.g. western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

In a further embodiment, the present invention provides the use of an antibody that specifically binds to at least one PTK7 polypeptide for screening for and/or diagnosis of carcinoma in a subject or for monitoring the efficacy of an anti- carcinoma therapy. The antibodies can be used, *inter alia*, for the diagnosis of carcinoma by detecting PTK7 expression in human tissue and/or in subfractions thereof, for example but without limitation, membrane, cytosolic or nuclear subfractions. These antibodies may be polyclonal, monoclonal, chimeric, humanised or bispecific or be single chains or fragments thereof.

Monoclonal antibodies are well known in the art and may be obtained by various techniques familiar to those skilled in the art (see, Kohler and Milstein, 1976, Eur. J. Immunol. 6: pp511-519). Chimeric antibodies are those antibodies encoded by immunoglobulin genes that have been genetically engineered so that the light and heavy chain genes are composed of immunoglobulin gene segments belonging to different species. These chimeric antibodies are likely to be less antigenic. Bispecific antibodies, may be made by methods known in the art, (Milstein *et al.*, 1983, Nature 305:537-539; WO 93/08829, Traunecker *et al.*, 1991, EMBO J. 10:3655-3659).

The antibodies in the present invention can also be generated using various phage display methods known in the art and include those disclosed by Brinkman *et al.* (in J.

Immunol. Methods, 1995, 182: 41-50), Ames *et al.* (in J. Immunol. Methods, 1995, 184:177-186), Kettleborough *et al.* (in Eur. J. Immunol. 1994, 24:952-958), Persic *et al.* (Gene, 1997 187 9-18), Burton *et al.*, (in Advances in Immunology, 1994, 57:191-280) and in WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108.

Such antibodies can be used for the diagnosis of carcinoma and/or to monitor or determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance for example, but without limitation, an enzyme (such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, acetylcholinesterase), a prosthetic group (such as streptavidin, avidin, biotin), a fluorescent material (such as umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrin), a luminescent material (such as luminol), a bioluminescent material (such as luciferase, luciferin, aequorin), a radioactive nuclide (such as ^{125}I , ^{131}I , ^{111}In , ^{99}Tc) a positron emitting metal or a non-radioactive paramagnetic metal ion (see U.S. 4,741,900).

In a further aspect, the method of detecting the presence of a PTK7 polypeptide comprises detecting the captured polypeptide using a directly or indirectly labelled detection reagent.

A PTK7 polypeptide can be detected by means of any immunoassay known in the art, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

The antibodies of the invention include analogues and derivatives that are modified, for example but without limitation, by the covalent attachment of any type of molecule. Preferably, said attachment does not impair immunospecific binding.

The invention also provides diagnostic kits comprising a capture reagent (e.g. an antibody) against a PTK7 polypeptide as defined above. In addition, such a kit may optionally comprise one or more of the following:

- (1) instructions for using the capture reagent for diagnosis, prognosis, therapeutic monitoring or any combination of these applications;
- (2) a labelled binding partner to the capture reagent;
- (3) a solid phase (such as a reagent strip) upon which the capture reagent is immobilised; and
- (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof.

If no labelled binding partner to the capture reagent is provided, the anti-polypeptide capture reagent itself can be labelled with a detectable marker, e.g., a chemiluminescent, enzymatic, fluorescent, or radioactive moiety (see above).

In another embodiment, the invention provides a method of screening for and/or diagnosis of carcinoma in a subject, which method comprises the step of detecting and/or quantifying the amount of a nucleic acid molecule in a biological sample obtained from said subject, wherein the nucleic acid molecule:

- d) comprises or consists of the DNA sequence shown in Figure 2 (SEQ ID NO: 2) or its RNA equivalent;
- e) has a sequence which is complementary to the sequences of d);
- f) has a sequence which codes for a polypeptide as defined in a) to c) above;
- g) has a sequence which shows substantial identity with any of those of d), e) and f); or
- h) is a fragment of d), e), f) or g), which is at least 8 nucleotides in length.

In view of the foregoing description the skilled person will appreciate that a large number of nucleic acids are within the scope of the present invention. Unless the context indicates otherwise, PTK7 nucleic acids include those nucleic acid molecules defined in d) to g) above and may have one or more of the following characteristics:

- 1) they may be DNA or RNA;
- 2) they may be single or double stranded;
- 3) they may be in substantially pure form. Thus, they may be provided in a form which is substantially free from contaminating proteins and/or from other nucleic acids; and
- 4) they may be with introns or without introns (e.g. as cDNA).

Fragments of PTK7 nucleic acids as described in 1) above are at least 8 nucleotides in length, preferably they are at least 20, at least 30, at least 50, at least 100 or at least 250 nucleotides in length.

The use of nucleic acid molecules that can hybridise to any of the nucleic acid molecules discussed above is also covered by the present invention. Such nucleic acid molecules are referred to as "hybridising" nucleic acid molecules. For example, but without limitation, hybridising nucleic acid molecules can be useful as probes or primers. A hybridising nucleic acid molecule of use in the present invention may have a high degree of sequence identity along its length with a nucleic acid molecule within the scope of d)-h) above (e.g. at least 50%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% sequence identity).

The PTK7 nucleic acid molecules comprising coding sequence for PTK7 polypeptides described above can be used for the recombinant production of said polypeptides for use in the present invention. The PTK7 nucleic acid molecules can additionally comprise coding sequence for example but without limitation, for a signal sequence (e.g. a signal peptide for secretion), or a leader sequence, a pro-sequence, a sequence to aid purification such as an affinity tag or an additional sequence conferring stability during production of the polypeptide.

If desired, a PTK7 nucleic acid molecule, can also be used in hybridisation assays. A PTK7 nucleic acid molecule comprising at least 8 nucleotides, can be used as a hybridisation probe. Hybridisation assays can be used for detection, prognosis, diagnosis, or monitoring of carcinoma. Accordingly, such a hybridisation assay comprises:

- i) contacting a patient sample containing nucleic acid with a nucleic acid probe capable of hybridising to a PTK7 nucleic acid molecule, under conditions such that hybridisation can occur; and
- ii) detecting or measuring any resulting hybridisation.

Preferably, such hybridising molecules are at least 10 nucleotides in length and are preferably at least 25 or at least 50 nucleotides in length. More preferably, the hybridising nucleic acid molecules specifically hybridise to nucleic acids within the scope of d), e), f), or g) above. Most preferably, the hybridisation occurs under stringent hybridisation conditions. One example of stringent hybridisation conditions is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution of about 0.9M. However, the skilled person will be able to vary such conditions as appropriate in order to take into account variables such as probe length, base composition, type of ions present, etc. For a high degree of selectivity, relatively stringent conditions such as low salt or high temperature conditions, are used to form the duplexes. Highly stringent conditions include hybridisation to filter-bound DNA in 0.5M NaHPO₄, 7% sodium dodecyl sulphate (SDS), 1mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). For some applications, less stringent conditions for duplex formation are required. Moderately stringent conditions include washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*). Hybridisation conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilise the hybrid duplex. Thus, particular hybridisation conditions can be readily manipulated, and will generally be chosen as appropriate. In general, convenient hybridisation temperatures in the presence of 50% formamide are: 42°C for a probe which is 95-100% identical to the fragment of a gene encoding a polypeptide as defined herein, 37°C for 90-95% identity and 32°C for 70-90% identity.

The invention also provides a diagnostic kit comprising a nucleic acid probe capable of hybridising to RNA encoding a PTK7 polypeptide, suitable reagents and instructions for use.

In a further embodiment, a diagnostic kit is provided comprising in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferably 10-30 nucleotides and still more preferably 10-20 nucleotides) that under appropriate reaction conditions can prime amplification of at least a portion of a PTK7 nucleic acid molecule, such as by polymerase chain reaction (see *e.g.*, Innis *et al.*, 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Q β replicase, cyclic probe reaction, or other methods known in the art.

Typically, primers are at least five nucleotides long and will generally be at least ten nucleotides long (e.g. fifteen to twenty-five nucleotides long). In some cases, primers of at least thirty or at least thirty-five nucleotides in length may be used.

A PTK7 polypeptide as described above can be useful as antigenic material, and may be used in the production of vaccines for treatment or prophylaxis of carcinoma. Such material can be antigenic and/or immunogenic. Antigenic includes a protein that is capable of being used to raise antibodies or indeed is capable of inducing an antibody response in a subject. Immunogenic material includes a protein that is capable of eliciting an immune response in a subject. Thus, in the latter case, the protein may be capable of not only generating an antibody response but, in addition, a non-antibody based immune responses i.e. a cellular or humoral response. It is well known in the art that it is possible to identify those regions of an antigenic or immunogenic polypeptide that are responsible for the antigenicity or immunogenicity of said polypeptide i.e. an epitope or epitopes. Amino acid and peptide characteristics well known to the skilled person can be used to predict the antigenic index (a measure of the probability that a region is antigenic) of a PTK7 polypeptide. For example, but without limitation, the 'Peptidestructure' program (Jameson and Wolf, 1988, CABIOS, 4(1):181) and a technique referred to as 'Threading' (Altuvia Y. *et al.*, 1995, J. Mol. Biol. 249:244) can be used. Thus, the PTK7 polypeptides may include one or more such epitopes or be sufficiently similar to such regions so as to retain their antigenic/immunogenic properties.

In another embodiment, the present invention provides the use of a PTK7 polypeptide, in the production of a composition for the treatment or prophylaxis of carcinoma, wherein the composition is a vaccine. The vaccine optionally comprises one or more suitable adjuvants or other vehicles. Examples of adjuvants well known in the art include inorganic gels, such as aluminium hydroxide, and water-in-oil emulsions, such as incomplete Freund's adjuvant. Other useful adjuvants will be well known to the skilled person.

Accordingly, in further embodiments, the present invention provides:

- A) the use of such a vaccine in inducing an immune response in a subject; and
- B) a method for the treatment or prophylaxis of carcinoma in a subject, or of vaccinating a subject against carcinoma which comprises the step of administering to the subject an effective amount of a PTK7 polypeptide, preferably as a vaccine.

In another embodiment, this invention provides a preparation of oligonucleotides comprising 10 or more consecutive nucleotides complementary to a PTK7 nucleic acid, for use as a vaccine for the treatment of carcinoma. Such a preparation can include adjuvants or other vehicles.

In a further embodiment, the invention provides a method of prophylaxis and/or treatment of carcinoma in a subject, comprising administering to a subject a therapeutically effective amount of a PTK7 polypeptide. In a specific embodiment, the PTK7 polypeptide for administration is a dominant negative mutant which is non-functional and inhibits the activity of endogenous PTK7.

In yet a further embodiment, the present invention provides a method for the prophylaxis and/or treatment of carcinoma in a subject, comprising administering to said

subject a therapeutically effective amount of at least one PTK7 nucleic acid molecule. In a specific embodiment, the nucleic acid encodes a dominant negative mutant of a PTK7 polypeptide. Preferably, the nucleic acid is administered via gene therapy (see for examples Hoshida, T. *et al.*, 2002, *Pancreas*, 25:111-121; Ikuno, Y. 2002, *Invest. Ophthalmol. Vis. Sci.* 2002 43:2406-2411; Bollard, C., 2002, *Blood* 99:3179-3187; Lee E., 2001, *Mol. Med.* 7:773-782).

In a specific embodiment, hybridising nucleic acid molecules are used as anti-sense molecules to alter the expression of PTK7 polypeptides by binding to complementary PTK7 nucleic acids. This technique can be used in anti-sense therapy. An antisense nucleic acid includes a nucleic acid capable of hybridising by virtue of some sequence complementarity to a portion of an RNA (preferably mRNA) encoding a PTK7 polypeptide. The antisense nucleic acid can be complementary to a coding and/or non-coding region of an mRNA encoding such a polypeptide. Such antisense nucleic acids have utility as compounds that inhibit expression, and can be used in the treatment or prevention of carcinoma. Most preferably, expression of a PTK7 polypeptide is inhibited by use of antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least eight nucleotides that are antisense to a gene or cDNA encoding a PTK7 polypeptide.

In one embodiment, the present invention provides the use of at least one PTK7 nucleic acid, in the preparation of a composition for use in the prophylaxis and/or treatment of carcinoma.

In another embodiment, the present invention provides the use of an antibody that binds to at least one PTK7 polypeptide, in the preparation of a composition for use in the prophylaxis and/or treatment of carcinoma. In particular, the preparation of vaccines and/or compositions comprising or consisting of antibodies is preferred. Most preferred are antibodies that bind specifically to PTK7 polypeptides. Thus, they can be used to deplete a sample comprising a PTK7 polypeptide of said polypeptide or to purify said polypeptide and/or inhibit the activity of such polypeptides.

In yet another embodiment, the present invention provides a method for the prophylaxis and/or treatment of carcinoma in a subject comprising administering to said subject, a therapeutically effective amount of at least one antibody that specifically recognises a PTK7 polypeptide. An antibody, optionally conjugated to a therapeutic moiety, can be used as a therapeutic composition that is administered alone or in combination with a cytotoxic factor(s) and/or cytokine(s). In particular, antibodies of the invention can be conjugated to a therapeutic agent or drug moiety to modify a given biological response. The therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a polypeptide possessing a desired biological activity. Such moieties may include, for example and without limitation, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumour necrosis factor, α -interferon, β -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony

stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), nerve growth factor (NGF) or other growth factor.

Techniques for conjugating such therapeutic moieties to antibodies are well known in the art (see, e.g., Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* eds., 1985 pp. 243-56, ed. Alan R. Liss, Inc; Hellstrom *et al.*, "Antibodies For Drug Delivery", in *Controlled Drug Delivery*, 2nd Ed., Robinson *et al.* eds., 1987, pp. 623-53, Marcel Dekker, Inc.; Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*; Pinchera *et al.*, 1985, eds., pp. 475-506; "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabelled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), 1985, pp. 303-16, Academic Press; Thorpe *et al.*, 1982 "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate (see U.S. 4,676,980).

In other embodiments, the invention provides fusion proteins of the antibodies (or functionally active fragments thereof), for example but without limitation, where the antibody or fragment thereof is fused via a covalent bond (e.g., a peptide bond), at optionally the N-terminus or the C-terminus, to an amino acid sequence of another protein (or portion thereof; preferably at least a 10, 20 or 50 amino acid portion of the protein). Preferably the antibody, or fragment thereof, is linked to the other protein at the N-terminus of the constant domain of the antibody. As stated above, such fusion proteins may facilitate depletion or purification of a polypeptide as described herein, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system.

In a further embodiment, the present invention provides methods for screening for anti- carcinoma agents that modulate the expression or activity of a PTK7 polypeptide or the expression of a PTK7 nucleic acid molecule. These agents may be useful in the treatment and/or prophylaxis of carcinoma.

In a further aspect, the present invention provides methods for screening for anti-carcinoma agents that interact with a PTK7 polypeptide or a PTK7 nucleic acid molecule.

The present invention provides methods for identifying agents (e.g., active agents or candidate agents) that are capable of modulating the expression or activity of a PTK7 polypeptide. Agents identified through the screening methods of the invention are potential therapeutics for use in the prophylaxis and/or therapy of carcinoma.

Examples of agents include but are not limited to, nucleic acids (e.g., DNA and RNA), carbohydrates, lipids, proteins, polypeptides, peptides, peptidomimetics, small molecules and other drugs. Agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to

peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, Anticancer Drug Des. 12:145; U.S. 5,738,996; and U.S. 5,807,683).

Examples of suitable methods based on the present description for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:6909; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann *et al.*, 1994, J. Med. Chem. 37:2678; Cho *et al.*, 1993, Science 261:1303; Carrell *et al.*, 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carrell *et al.*, 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop *et al.*, 1994, J. Med. Chem. 37:1233.

Libraries of compounds may be presented, for example, presented in solution (e.g., Houghten, 1992, Bio/Techniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (US 5,223,409), spores (US 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:1865-1869) or phage (Scott and Smith, 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; and Felici, 1991, J. Mol. Biol. 222:301-310).

In one embodiment, agents that modulate the expression of a polypeptide are identified in a cell-based assay system. Accordingly, cells expressing a PTK7 polypeptide are contacted with a candidate agent or a control agent and the ability of the candidate agent to alter expression of the PTK7 polypeptide is determined. In a further embodiment, the expression of the PTK7 polypeptide may be compared to a reference range or control. If desired, this assay may be used to screen a plurality (e.g. a library) of candidate agents. The cell, for example, can be of prokaryotic origin (e.g., *E. coli*) or eukaryotic origin (e.g., yeast or mammalian). Further, the cells can express a PTK7 polypeptide endogenously or be genetically engineered to express a PTK7 polypeptide. The ability of the candidate agents to alter the expression of a PTK7 polypeptide can be determined by methods known to those of skill in the art, for example, by flow cytometry, a scintillation assay, immunoprecipitation or western blot analysis.

In another embodiment, agents that modulate the expression of a PTK7 polypeptide are identified in a cell-free assay system. In accordance with this embodiment, an expression vector comprising a PTK7 nucleic acid is contacted with a candidate agent or a control agent and the ability of the candidate agent to modulate the expression of a PTK7 polypeptide is determined. In a further embodiment, the expression of the PTK7 nucleic acid is compared to a reference range or control. If desired, this assay may be used to screen a plurality (e.g. a library) of candidate agents.

In another embodiment, a cell-based assay system is used to identify agents capable of modulating the activity of a PTK7 polypeptide. In such an assay, the activity of a PTK7 polypeptide is measured in population of cells that naturally or recombinantly express a PTK7 polypeptide, in the presence of a candidate agent and in the absence of a candidate agent (e.g. in the presence of a control agent) and the activity of the PTK7 polypeptide is compared. The candidate agent can then be identified as a stimulator or inhibitor of the activity of a PTK7 polypeptide based on this comparison. In a further embodiment, a cell-free

assay can be used to measure the activity of a PTK7 polypeptide as defined above, in the presence or absence of a candidate agent. In a further embodiment, the activity of a PTK7 polypeptide is compared to a reference range or control.

In another embodiment, a cell-free assay system is used to identify agents such as an enzyme, or a biologically active portion thereof, which is responsible for the production or degradation of a PTK7 polypeptide, or is responsible for the post-translational modification of a PTK7 polypeptide. In a primary screen, a plurality (*e.g.*, a library) of agents are contacted with cells that naturally or recombinantly express: (i) a PTK7 polypeptide, and (ii) a protein that is responsible for processing of a PTK7 polypeptide, in order to identify agents that modulate the production, degradation, or post-translational modification of a PTK7 polypeptide. If desired, agents identified in the primary screen can then be assayed in a secondary screen against cells naturally or recombinantly expressing the PTK7 polypeptide. The ability of the candidate agent to modulate the production, degradation or post-translational modification of a PTK7 polypeptide can be determined by methods known to those of skill in the art, including without limitation, flow cytometry, a scintillation assay, kinase assay, phosphatase assay, immunoprecipitation and western blot analysis.

In one embodiment, agents that modulate the expression of a polypeptide are identified by contacting cells (*e.g.*, cells of prokaryotic origin or eukaryotic origin) expressing a PTK7 polypeptide with a candidate agent or a control agent (*e.g.*, phosphate buffered saline; PBS) and determining the expression of a PTK7 polypeptide or mRNA encoding a PTK7 polypeptide. The level of expression of a PTK7 polypeptide or mRNA encoding said polypeptide in the presence of the candidate agent is compared to the level of expression of a PTK7 polypeptide or mRNA encoding said polypeptide in the absence of the candidate agent (*e.g.*, in the presence of a control agent). The candidate agent can then be identified as a modulator of the expression of a PTK7 polypeptide based on this comparison. For example, when expression of a PTK7 polypeptide (or its mRNA) is significantly greater in the presence of the candidate agent than in its absence, the candidate agent is identified as a stimulator of expression of a PTK7 polypeptide. Alternatively, when expression of a PTK7 polypeptide (or its mRNA) is significantly less in the presence of the candidate agent than in its absence, the candidate agent is identified as an inhibitor of the expression of polypeptide. The level of expression of a PTK7 polypeptide or its encoding mRNA can be determined by methods known to those of skill in the art. For example, mRNA expression can be assessed by Northern blot analysis or RT-PCR, and protein levels can be assessed, without limitation, by western blot analysis.

In another embodiment, agents that modulate the expression of a PTK7 polypeptide are identified in an animal model. Examples of suitable animals include, but are not limited to, mice, rats, rabbits, monkeys, guinea pigs, dogs and cats. Preferably, the animal used represents a model of carcinoma. In accordance with this embodiment, the candidate agent or a control agent is administered (*e.g.*, orally, rectally or parenterally such as intraperitoneally or intravenously) to a suitable animal and the effect on the expression of a PTK7 polypeptide (or its mRNA) is determined. Changes in the expression of a polypeptide can be assessed by the methods outlined above.

In yet another embodiment, a PTK7 polypeptide is used as a "bait protein" in a two-hybrid assay or three hybrid assay to identify other proteins that bind to or interact with the polypeptide (see, e.g., U.S. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Bio/Techniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and WO 94/10300). As those skilled in the art will appreciate, such binding proteins are also likely to be involved in the propagation of signals by a PTK7 polypeptide, for example, they may be upstream or downstream elements of a signalling pathway involving a PTK7 polypeptide.

One skilled in the art will also appreciate that a polypeptide may also be used in a method for the structure-based design of an agent, in particular a small molecule which acts to modulate (e.g. stimulate or inhibit) the activity of said polypeptide, said method comprising:

- 1) determining the three-dimensional structure of said polypeptide;
- 2) deducing the three-dimensional structure of the likely reactive or binding site(s) of the agent;
- 3) synthesising candidate agents that are predicted to react or bind to the deduced reactive or binding site; and
- 4) testing whether the candidate agent is able to modulate the activity of said polypeptide.

It will be appreciated that the method described above is likely to be an iterative process.

This invention further provides novel active agents identified by the above-described screening methods and uses thereof for treatments as described herein.

As discussed herein, active agents of the invention find use in the treatment or prophylaxis of carcinoma.

Thus, in an additional embodiment, the present invention provides a pharmaceutical composition comprising at least one active agent of the invention, optionally together with one or more pharmaceutically acceptable excipients, carriers or diluents. In one aspect, the pharmaceutical composition is for use as a vaccine and so any additional components will be acceptable for vaccine use. In addition, the skilled person will appreciate that one or more suitable adjuvants may be added to such vaccine preparations.

Pharmaceutical formulations may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such formulations may be prepared by any method known in the art of pharmacy, for example by bringing into association the active ingredient with the carrier(s) or excipient(s).

Pharmaceutical formulations adapted for oral administration may be presented as discrete units such as capsules or tablets; powders or granules; solutions or suspensions in aqueous or non-aqueous liquids; edible foams or whips; or oil-in-water liquid emulsions or water-in-oil liquid emulsions.

Pharmaceutical formulations adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3(6), 318, (1986).

Pharmaceutical formulations adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, impregnated dressings, sprays, aerosols or oils and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

For applications to the eye or other external tissues, for example the mouth and skin, the formulations are preferably applied as a topical ointment or cream. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base.

Pharmaceutical formulations adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent.

Pharmaceutical formulations adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

Pharmaceutical formulations adapted for rectal administration may be presented as suppositories or enemas.

Pharmaceutical formulations adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns. Suitable formulations wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient.

Pharmaceutical formulations adapted for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurised aerosols, nebulizers or insufflators.

Pharmaceutical formulations adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical formulations adapted for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration.

Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations may also include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrrolidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tableting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated according to methods well known in normal pharmaceutical practice.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives, such as suspending agents, for example sorbitol, methyl cellulose, glucose syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example almond oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl *p*-hydroxybenzoate or sorbic acid, and, if desired, conventional flavouring or colouring agents.

Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, can be either suspended or dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be

supplied to reconstitute the liquid prior to use. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilization cannot be accomplished by filtration. The compound can be sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent.

The compositions may contain from 0.1% by weight, preferably from 10-60% by weight, of the active material, depending on the method of administration.

Pharmaceutical formulations may be presented in unit dose forms containing a predetermined amount of active ingredient per dose. Such a unit may contain for example 100mg/kg to 1mg/kg depending on the condition being treated, the route of administration and the age, weight and condition of the patient. Preferred unit dosage formulations are those containing a daily dose or sub-dose, as herein above recited, or an appropriate fraction thereof, of the active ingredient.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a formula (I) compound will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular mammal being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of the compound of formula (I) given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Dosage regimens are adjusted to provide the optimum desired response (e. g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Pharmaceutical compositions of the invention also can be administered in combination therapy, i.e., combined with other agents. For example, the combination therapy

can include a composition of the present invention with at least one anti-tumour agent or other conventional therapy.

In certain embodiments, the antibodies of the invention can be formulated to ensure proper distribution in vivo, for example, in liposomes. For methods of manufacturing liposomes, see, e. g., U. S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e. g., V. V. Ranade (1989) J. Clin. Pharmacol. 29: 685).

Exemplary targeting moieties include folate or biotin (see, e. g., U. S. Patent 5,416,016); mannosides (Umezawa et al., (1988) Biochem. Biophys. Res. Commun. 153: 1038); antibodies (P. G. Bloeman et al. (1995) FEBSLett. 357: 140; M. Owais et al. (1995) Antimicrob. Agents Chemother. 39: 180); surfactant protein A receptor (Briscoe et al. (1995) Am. J. Physiol. 1233: 134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; psi 20 (Schreier et al. (1994) J. Biol. Chem. 269: 9090); see also K. Keinänen; M. L. Laukkanen (1994) FEBSLett. 346: 123; J. J. Killian; I. J. Fidler (1994) Immunomethods 4 : 273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor.

The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

In a further embodiment, the present invention provides a method for the prophylaxis and/or treatment of carcinoma in a subject, which comprises administering to said subject a therapeutically effective amount of at least one active agent of the invention.

Another aspect of the invention provides the use of at least one active agent in the preparation of a composition for the treatment of carcinoma.

In the context of the present invention, the biological sample can be obtained from any source, such as a serum sample or a tissue sample, e.g. colon tissue:

Preferred features of each embodiment of the invention are as for each of the other embodiments *mutatis mutandis*. The prior art documents mentioned herein are incorporated by reference to the fullest extent permitted by law.

The invention will now be described with reference to the following examples, which should not in any way be construed as limiting the scope of the present invention. The examples refer to the figures in which:

Figure 1 shows the protein sequence of PTK7 (JC4593/U40271), SEQ ID NO:1. The tandem mass spectrum peptides are in bold and underlined, MALDI mass spectra peptides are in bold.

Figure 2 shows the nucleic acid sequence of PTK7 (JC4593/U40271), SEQ ID NO: 2.

Figure 3 shows the distribution of PTK7 mRNA in normal tissues. mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA.

Figure 4 shows the distribution of PTK7 mRNA in patient matched adjacent normal and tumour breast tissues, mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA.

Figure 5 shows the distribution of PTK7 mRNA in breast tumour tissues. 40 tumour samples were obtained from patients with (A) or without (B) lymph node metastasis. Ethical approval for the normal and tumour breast samples was obtained at surgery (University of Oxford, UK). mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA. Note that PTK7 mRNA expression was significantly elevated in samples from patients with lymph node metastasis relative to both samples from patients with no lymph node metastases and normal tissues.

Figure 6 shows the distribution of PTK7 mRNA in matched normal and tumour lung tissues, mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA.

Figure 7 shows the distribution of PTK7 mRNA in matched normal and tumour kidney tissues, mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA.

Figure 8 shows the distribution of PTK7 mRNA in matched normal and tumour pancreatic tissues, mRNA levels were quantified by real time RT-PCR and are expressed as the number of copies ng⁻¹ cDNA.

Example 1 – isolation of PTK7 protein from lung and liver cell lines:

Proteins in lung and liver cancer cell line membranes were separated by SDS-PAGE and analysed.

Crude fractionation of lung and liver cancer cell lines***1a - cell culture***

The lung cancer cell lines DMS144 and SHP-77 were cultured in EMEM plus 1% NEAA media, supplemented with 10% foetal calf serum, 2mM glutamine, 1% penicillin and 1% streptomycin. The liver cancer cell line HepG2 was cultured in EMEM plus 1% NEAA media, supplemented with 10% foetal calf serum, 2mM glutamine, 1% penicillin and 1% streptomycin. The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

1b - Cell fractionation and plasma membrane generation

Purified membrane preparations were isolated from the cell lines. Adherent cells (2×10^8) were washed three times with PBS and scraped using a plastic cell lifter. Cells were centrifuged at 1000 x g for 5 min at 4°C and the cell pellet was resuspended in homogenisation buffer (250 mM Sucrose, 10mM HEPES, 1mM EDTA, 1mM Vanadate and 0.02% azide, protease inhibitors). Cells were fractionated using a ball bearing homogeniser (8.002 mm ball, HGM Lab equipment) until approx. 95% of cells were broken. Membranes were fractionated using the method described by Pasquali *et al* (Pasquali C. *et al.*, 1999 J. Chromatography 722: pp 89-102). The fractionated cells were centrifuged at 3000 x g for 10 min at 4°C and the postnuclear supernatant was layered onto a 60% sucrose cushion and centrifuged at 100 000 x g for 45 min. The membranes were collected using a pasteur pipette and layered on a preformed 15 to 60% sucrose gradient and spun at 100 000 x g for 17 hours. Proteins from the fractionated sucrose gradient were run on a 4-20% 1D gel (Novex) and subject to western blotting; those fractions containing alkaline phosphatase and transferrin immunoreactivity but not oxidoreductase II or calnexin immunoreactivity were pooled and represented the plasma membrane fraction.

1c - Preparation of plasma membrane fractions for 1D-gel analysis

Plasma membrane fractions that had transferrin immunoreactivity but no oxidoreductase II or calnexin immunoreactivity were identified. These sucrose fractions were pooled and diluted at least four times with 10mM HEPES, 1mM EDTA 1mM Vanadate, 0.02% Azide. The diluted sucrose fraction was added to a SW40 or SW60 tube and centrifuged at 100 000 x g for 45 minutes with slow acceleration and deceleration. The supernatant was removed from the membrane pellet and the pellet washed three times with PBS-CM. The membrane pellet was solubilised in 2% SDS in 63mM TrisHCl, pH 7.4. A protein assay was performed followed by the addition of mercaptoethanol (2% final), glycerol

(10%) and bromophenol blue (0.0025% final) was added. A final protein concentration of 1 microgram/microlitre was used for 1D-gel loading.

1d - 1D-gel technology

Protein or membrane pellets were solubilised in 1D-sample buffer (approximately 1 mg/ml) and the mixture heated to 95°C for 5 min.

Samples were separated using 1D-gel electrophoresis on pre-cast 8-16% gradient gels purchased from Bio-Rad (Bio-Rad Laboratories, Hemel Hempstead, UK). A sample containing 30-50 micrograms of the protein mixtures obtained from a detergent extract were applied to the stacking gel wells using a micro-pipette. A well containing molecular weight markers (10, 15, 25, 37, 50, 75, 100, 150 and 250 kDa) was included for calibration by interpolation of the separating gel after imaging. Separation of the proteins was performed by applying a current of 30mA to the gel for approximately 5 hours or until the bromophenol blue marker dye had reached the bottom of the gel.

After electrophoresis the gel plates were prised open, the gel placed in a tray of fixer (10% acetic acid, 40% ethanol, 50% water) and shaken overnight. The gel was then primed for 30 minutes by shaking in a primer solution (7.5% acetic acid, 0.05% SDS in Milli-Q water) followed by incubation with a fluorescent dye (0.06% OGS dye in 7.5% acetic acid) with shaking for 3 hrs. A preferred fluorescent dye is disclosed in US Patent No. 6,335,446. Sypro Red (Molecular Probes, Inc., Eugene, Oregon) is a suitable alternative dye for this purpose.

A digital image of the stained gel was obtained by scanning on a Storm Scanner (Molecular Dynamics Inc, USA) in the blue fluorescence mode. The captured image was used to determine the area of the gel to excise for in-gel proteolysis.

1e - Recovery and analysis of selected proteins

Each vertical lane of the gel was excised using either a stainless steel scalpel blade or a PEEK gel cutter (OGS) that cuts sequentially down the length of the gel lane with no attempt at collecting specific protein bands.

Proteins were processed using in-gel digestion with trypsin (Modified trypsin, Promega, Wisconsin, USA) to generate tryptic digest peptides. Recovered samples were divided into two. Prior to MALDI analysis samples were desalted and concentrated using C18 Zip Tips™ (Millipore, Bedford, MA). Samples for tandem mass spectrometry were purified using a nano LC system (LC Packings, Amsterdam, The Netherlands) incorporating C18 SPE material. Recovered peptide pools were analysed by MALDI-TOF-mass spectrometry (Voyager STR, Applied Biosystems, Framingham, MA) using a 337 nm wavelength laser for desorption and the reflectron mode of analysis. Pools were also analyzed by nano-LC tandem mass spectrometry (LC/MS/MS) using a Micromass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass, Altrincham, UK). For partial amino acid sequencing and identification of liver and lung cancer cell membrane proteins uninterpreted tandem mass spectra of tryptic peptides were searched against a database of public domain proteins constructed of protein entries in the non-redundant

database held by the National Centre for Biotechnology Information (NCBI) which is accessible at <http://www.ncbi.nlm.nih.gov/> using the SEQUEST search program (Eng *et al.*, 1994, J. Am. Soc. Mass Spectrom. 5:976-989), version v.C.1. Criteria for database identification included: the cleavage specificity of trypsin; the detection of a suite of a, b and y ions in peptides returned from the database, and a mass increment for all Cys residues to account for carbamidomethylation. Following identification of proteins through spectral-spectral correlation using the SEQUEST program, masses detected in MALDI-TOF mass spectra were assigned to tryptic digest peptides within the proteins identified. In cases where no amino acid sequences could be identified through searching with uninterpreted MS/MS spectra of tryptic digest peptides using the SEQUEST program, tandem mass spectra of the peptides were interpreted manually, using methods known in the art. (In the case of interpretation of low-energy fragmentation mass spectra of peptide ions see Gaskell *et al.*, 1992, Rapid Commun. Mass Spectrom. 6:658-662). The method described in WO 02/21139 was also used to interpret mass spectra.

Four tandem spectra (shown in bold and underlined in Figure 1) and 16 mass matches (shown in bold in Figure 1) were found to match the GenBank accession numbers J05257 and U40271.

Example 2: Normal tissue distribution and disease tissue upregulation of PTK7 using quantitative RT-PCR (Taqman) analysis

Real time RT-PCR was used to quantitatively measure PTK7 expression in a range of tumour tissues and matched controls. Ethical approval for the normal and tumour breast samples was obtained at surgery (University of Oxford, UK). The lung, pancreatic and kidney tumour samples were obtained from Clinomics Inc. The primers used for PCR were as follows:

Sense, 5'- cagccagaacttcaccttgagc – 3', (SEQ ID NO: 3)

Antisense, 5'- catgggagtcctcctcaaag– 3' (SEQ ID NO: 4)

Reactions containing 5ng cDNA, SYBR green sequence detection reagents (PE Biosystems) and sense and antisense primers were assayed on an ABI7700 sequence detection system (PE Biosystems). The PCR conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15s, 60°C for 1min. The accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence, and the data were analysed using the Sequence Detector program v1.6.3 (PE Biosystems). Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were used to calculate PTK7 copy number in each sample.

Relatively low expression levels of PTK7 were seen in normal tissues (less 2000 copies per ng⁻¹ cDNA, Figure 3).

In contrast, levels of PTK7 expression were greatly increased in breast tumour samples relative to their matched controls with 8/23 samples showing expression levels of greater than 2500 copies per ng-1 cDNA (Figure 4). Furthermore, a large upregulation in PTK7 expression could be seen in 9/20 samples from patients with lymph node metastases (>50,000 copies per ng⁻¹ cDNA) compared to both normal tissue and tissue from patients with no lymph node metastases (Figure 5).

The expression of PTK7 expression was examined in 7 lung tumour samples and an increased expression was seen in all seven samples relative to a control sample of unaffected lung (Figure 6). PTK7 mRNA expression was also increased in 8/9 kidney tumour samples relative to either control kidney tissue or a matched control sample (Figure 7) and in 6/6 pancreatic tumour samples relative to normal tissue (Figure 8).

This data suggests that PTK7 is increased in a selection of carcinomas including breast cancer, lung cancer, kidney cancer and pancreatic cancer. In addition, a large increase in PTK7 expression is seen in 45% of samples from breast cancer patients with lymph node metastases relative to both normal tissue and samples from breast cancer patients with no lymph node metastases, indicating that PTK7 is likely to be of utility as a carcinoma target.

CLAIMS

1. A method of screening for and/or diagnosis of carcinoma in a subject, and/or monitoring the effectiveness of carcinoma therapy, which comprises the step of detecting and/or quantifying in a biological sample obtained from said subject:
 - (i) a PTK7 polypeptide which:
 - a) comprises or consists of the amino acid sequence shown in Figure 1 (SEQ ID NO:1);
 - b) is a derivative having one or more amino acid substitutions, modifications, deletions or insertions relative to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1) which retains the activity of PTK7; or
 - c) is a fragment of a polypeptide having the amino acid sequence shown in Figure 1 (SEQ ID NO: 1), which is at least ten amino acids long and has at least 70% homology over the length of the fragment;
 - (ii) a nucleic acid molecule which:
 - d) comprises or consists of the DNA sequence shown in Figure 2 (SEQ ID NO: 2) or its RNA equivalent;
 - e) has a sequence which is complementary to the sequences of d);
 - f) has a sequence which codes for a polypeptide as defined in any of a) to c) above;
 - g) has a sequence which shows substantial identity with any of those of d), e) and f); or
 - h) is a fragment of d), e), f) or g), which is at least 8 nucleotides in length.
2. The method of claim 1, wherein the level of said polypeptide or said nucleic acid is compared to a previously determined reference range or control.
3. The method according to claim 1, wherein the step of detecting comprises:
 - (a) contacting the sample with a capture reagent that is specific for a polypeptide as defined in claim 1(i); and
 - (b) detecting whether binding has occurred between the capture reagent and said polypeptide in the sample.

4. The method according to claim 3, wherein step (b) comprises detecting the captured polypeptide using a directly or indirectly labelled detection reagent.
5. An antibody, functionally-active fragment, derivative or analogue thereof, that specifically binds to one or more PTK7 polypeptides as defined in claim 1(i).
6. The method according to anyone of claims 1 to 4, wherein a PTK7 polypeptide is detected and/ or quantified using an antibody that specifically binds to one or more PTK7 polypeptides as defined in claim 1(i).

7. An antibody according to claim 5 or the method of claim 6, wherein the antibody is monoclonal, polyclonal, chimeric, humanised or bispecific, or is conjugated to a therapeutic moiety, detectable label, second antibody or a fragment thereof, a cytotoxic agent or cytokine.
8. The method according to claim 3 or 4, wherein the capture reagent is immobilised on a solid phase.
9. A diagnostic kit comprising a capture reagent specific for a PTK7 polypeptide as defined in claim 1(i), reagents and instructions for use.
10. The kit of claim 9, wherein the capture reagent is an antibody.
11. The kit of claim 10, wherein the antibody is monoclonal.
12. A method for the prophylaxis and/ or treatment of carcinoma in a subject, which comprises administering to said subject a therapeutically effective amount of
 - (i) at least one PTK7 polypeptide as defined in claim 1(i),
 - (ii) a nucleic acid molecule as defined in claim 1(ii),
 - (iii) an antibody as defined in claims 5 or 7, or
 - (iv) a derivative of a polypeptide as defined in claim 1(i) having one or more amino acid substitutions, modifications, deletions or insertions relative to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1) which is a dominant negative mutant.
13. The use of:
 - (i) at least one PTK7 polypeptide as defined in claim 1(i),
 - (ii) a nucleic acid molecule as defined in claim 1(ii),
 - (iii) a composition comprising an antibody as defined in claims 5 or 7, or
 - (iv) a derivative of a polypeptide as defined in claim 1(i) having one or more amino acid substitutions, modifications, deletions or insertions relative to the amino acid sequence shown in Figure 1 (SEQ ID NO: 1) which is a dominant negative mutant.in the prophylaxis and/ or treatment of carcinoma.
14. The use as claimed in claim 13(i) or (iv), wherein the composition is a vaccine.
15. A method of screening for anti-carcinoma agents that interact with a polypeptide as defined in claim 1(i), said method comprising:
 - (a) contacting said polypeptide with a candidate agent; and
 - (b) determining whether or not the candidate agent interacts with said polypeptide.

16. The method according to claim 15, wherein the determination of interaction between the candidate agent and PTK7 polypeptide comprises quantitatively detecting binding of the candidate agent and said polypeptide.

17. A method of screening for anti-carcinoma agents that modulate

- (a) the expression or activity of a PTK7 polypeptide as defined in claim 1(i), or
- (b) the expression of a nucleic acid molecule as defined in claim 1(ii),

comprising

- (i) comparing the expression or activity of said polypeptide, or the expression of said nucleic acid molecule, in the presence of a candidate agent with the expression or activity of said polypeptide, or the expression of said nucleic acid molecule, in the absence of the candidate agent or in the presence of a control agent; and
- (ii) determining whether the candidate agent causes the expression or activity of said polypeptide, or the expression of said nucleic acid molecule, to change.

18. The method of claim 17, wherein the expression or activity level of said polypeptide, or the expression level of said nucleic acid molecule is compared with a predetermined reference range.

19. The method of claim 17 or 18, wherein part (ii) additionally comprises selecting an agent which modulates the expression or activity of said polypeptide, or the expression of said nucleic acid molecule for further testing, or therapeutic or prophylactic use as an anti-carcinoma agent.

20. An active agent identified by the method of any of claims 15-19, which causes the expression or activity of said polypeptide, or the expression of said nucleic acid molecule, to change.

21. The use of an active agent according to claim 20 in the manufacture of a composition for the prophylaxis and/or treatment of carcinoma.

22. An active agent according to claim 20 for use in the prophylaxis and/or treatment of carcinoma.

23. A method of prophylaxis and/or treatment of carcinoma, which comprises administering to said subject a therapeutically effective amount of an active agent according to claim 20.

Figure 1

1 MGAARGSPAR PRRLPLLSVL LLPLLGGTQT AIVFIKQPSS QDALQGRRAL LRCEVEAPGP
 61 VHVVWLLDGA PVQDTERRFA QGSSLSFAAV DRLQDSGTFQ CVARDDVTGE EARSANASFN
 121 IKWIEAGPVV LKHPASEAEI QPQTQVTLRC HIDGHPRTY QWFRDGTPLS DGQSNHTVSS
 181 KERNLTLRPA GPEHSGLYSC CAHSAFGQAC SSQNFTLSIA DESFARVVLA PODVVVARYE
 241 EAMFHCQFSA QPPPSLQWLF EDETPITNRS RPPHLRRATV FANGSLLLTQ VRPRNAGIYR
 301 CIGQGQRGPP IILEATLHLA EIEDMPLFEP RVFTAGSEER VTCLPPKGLP EPSVWWEHAG
 361 VRLPTHGRVY QKGHELVLAN IAESDAGVYT CHAANLAGQR RQDVNITVAT VPSWLKKPQD
 421 SQLEEGKPGY LDCLTQATPK PTVVWYRNQM LISEDSRFEV FKNGTLRINS VEYDGTWYR
 481 CMSSTPAGSI EAQARVQVLE KLKFTPPPQP QQCMEFDKEA TVPCSATGRE KPTIKWERAD
 541 GSSLPEWVTD NAGTLHFARV TRDDAGNYTC IASNGPQGQI RAHVQLTVAV FITFKVEPER
 601 TTVYQGHTAL LQCEAQGDPK PLIQWKGKDR ILDP TKLGPR MHIFQNGSLV IHDVAPEDSG
 661 RYTCIAGNSC NIKHTEAPLY VVDKPVPES EGP GSPPPYK MIQTIGLSVG AAVAYIIAVL
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 841 LDFRRELEMF GKLNHANVVR LLGLCREAEP HYMVLEYVDL GDLKQFLRIS KSKDEKLKSQ
 901 PLSTKQKVAL CTQVALGMEH LSNNRFVHKD LAARNCLVSA QRQVKVSALG LSKDVYNSEY
 961 YHFRQAWVPL RWMSPEAILE GDFSTKSDVW AFGVLMWEVF THGEMPHGGQ ADDEV LADLQ
 1021 AGKARLPQPE GCP SKLYRLM QRCWALSPKD RPSFSEIASA LGDSTVDSKP

Figure 2

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Figure 3

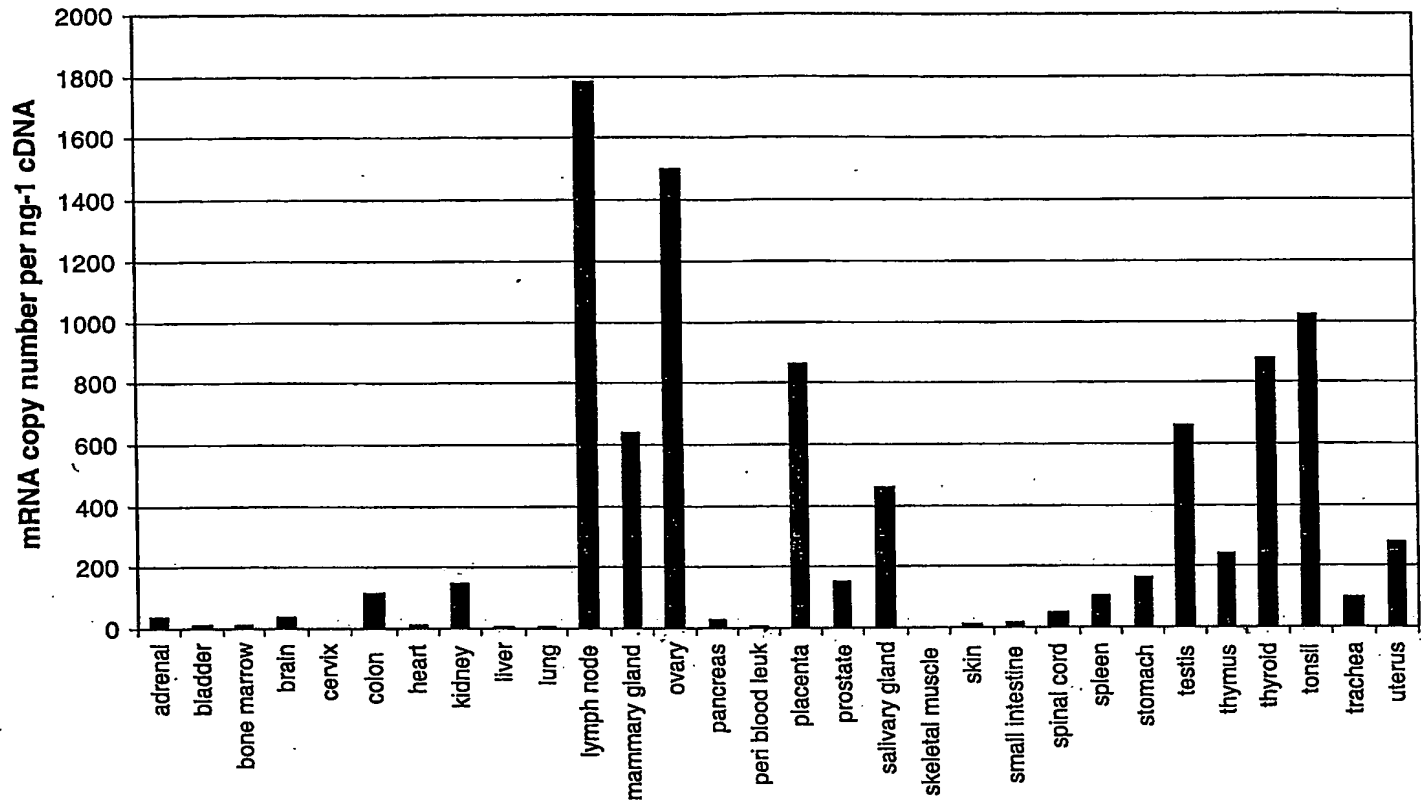


Figure 4

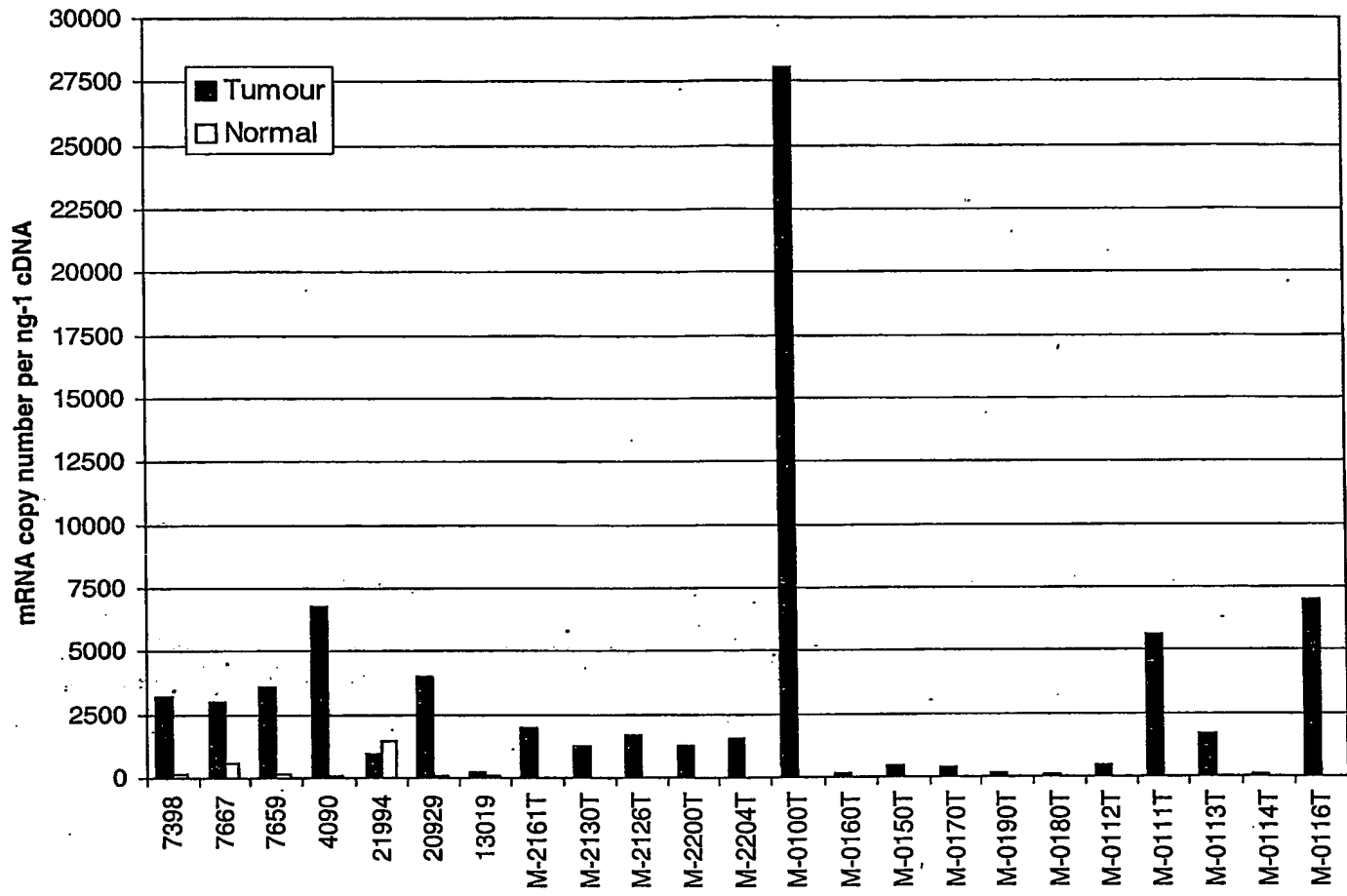


Figure 5

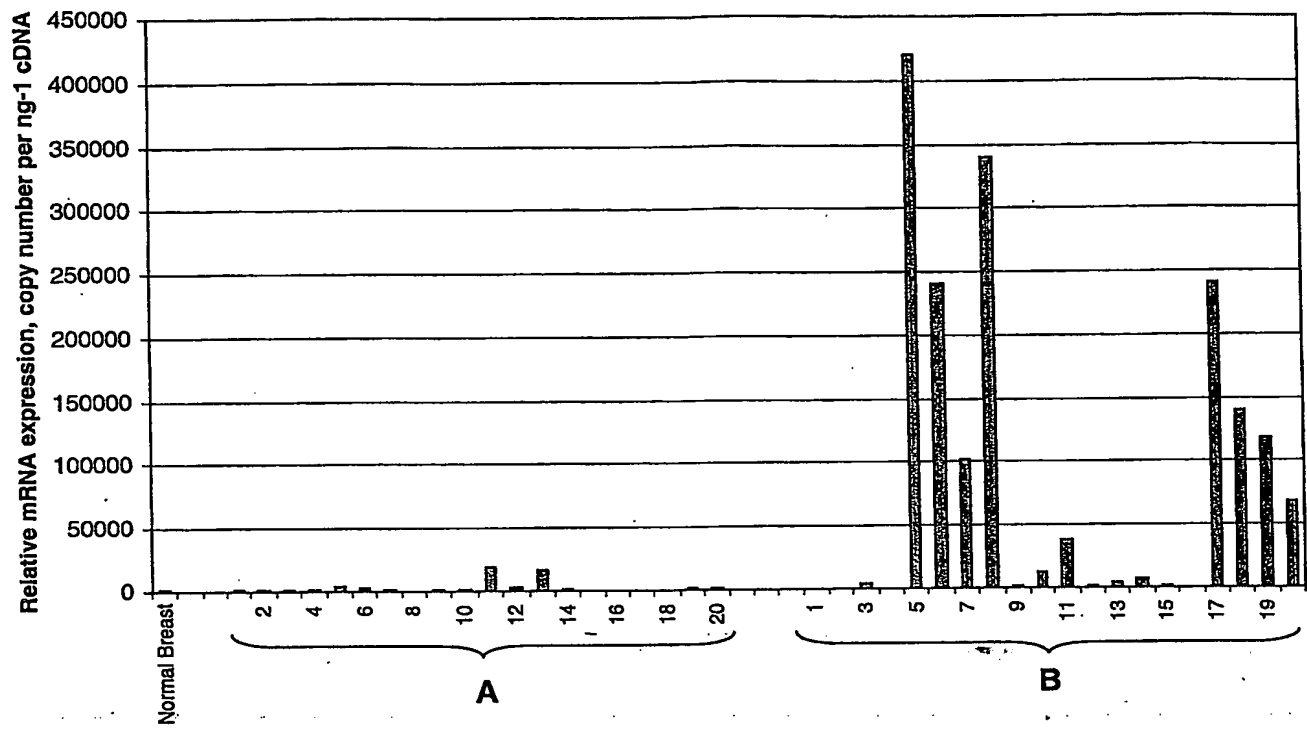


Figure 6

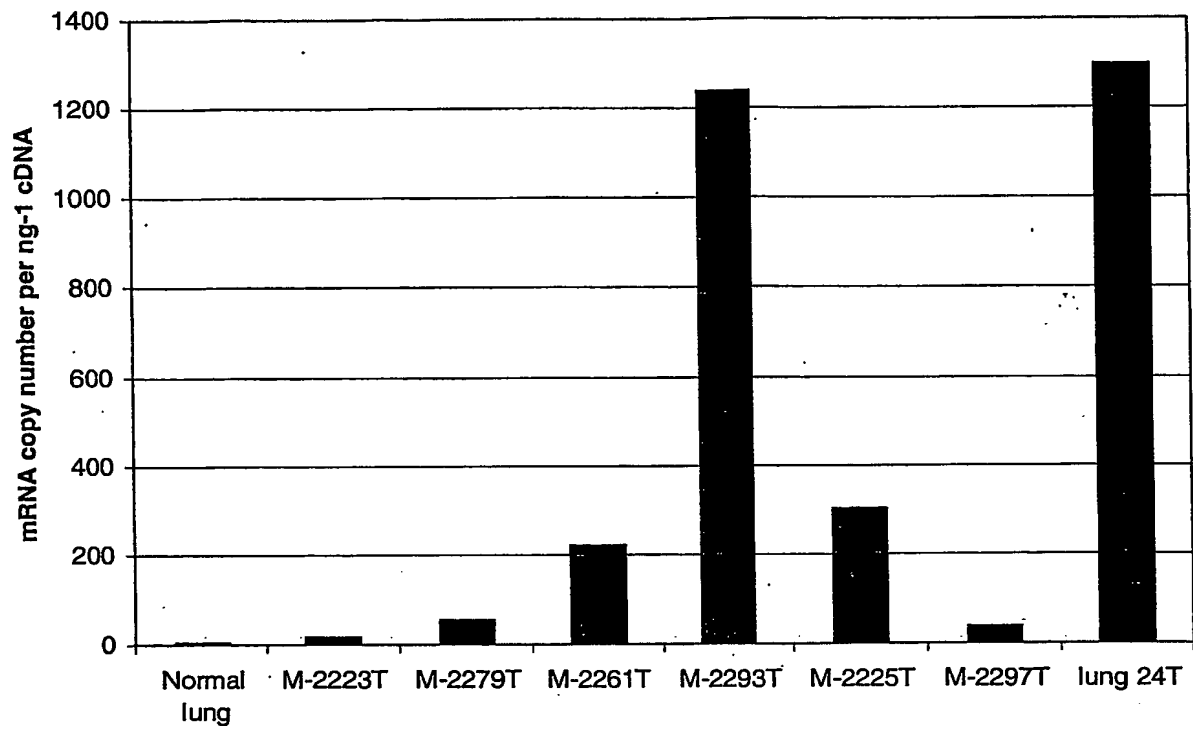


Figure 7

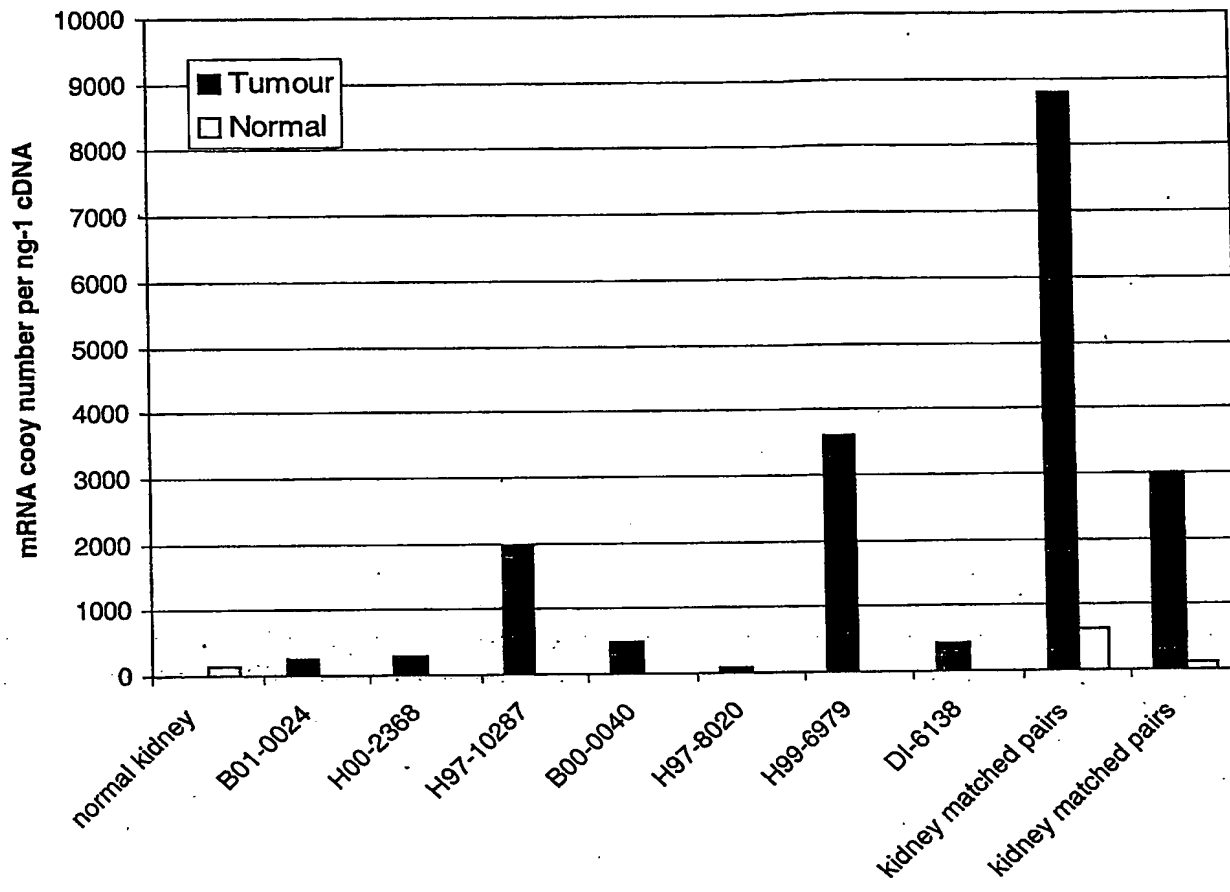
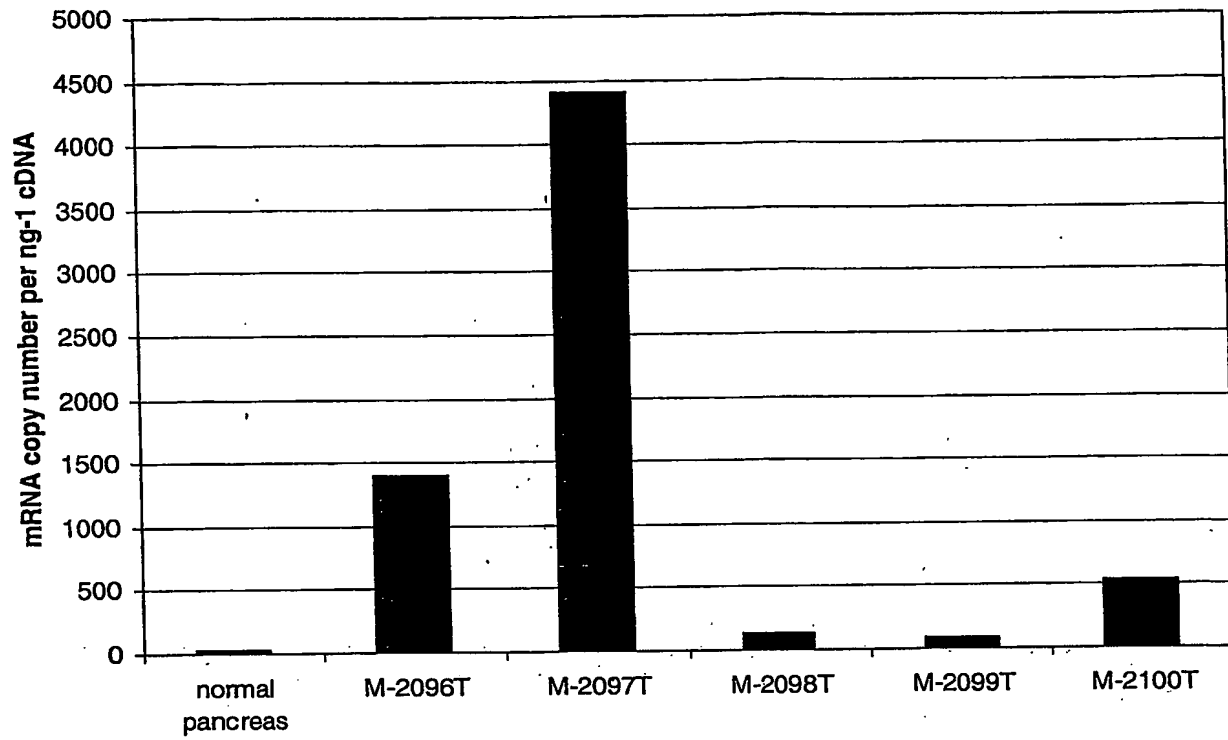


Figure 8



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